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DOI: https://doi.org/10.1111/j.1574-6941.1997.tb00406.x

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FEMS Microbiology Ecology 23 (1997) 239–247

FEMS MICROBIOLOGY ECOLOGY

Gene transfer between bacteria within digestive vacuoles of protozoa

Wolfram Schlimme [a], Marcello Marchiani [a], Kurt Hanselmann [b], Bernard Jenni [a,*]

[a] *Novartis Pharma AG, K-135.1.17, CH-4002 Basel, Switzerland*

[b] *Institute of Plant Biology, Dept. of Microbiology, University of Zürich, CH-8008 Zürich, Switzerland*

Received 21 November 1996; revised 25 March 1997; accepted 28 April 1997

Abstract

The occurrence of horizontal gene transfer between bacteria within digestive vacuoles and faecal pellets of the protozoan *Tetrahymena pyriformis* was investigated. More than 90% of the egested faecal pellets of *T. pyriformis*, added as predator to a suspension of *Escherichia coli*, contained viable bacteria. In a mixed population, containing donor (plasmid RP4) and recipient *E. coli* cells, the presence of *T. pyriformis* increased conjugational gene transfer by three orders of magnitude. Since the protozoa formed an average of 12–13 digestive vacuoles per cell, each protozoan had statistically egested one or more transconjugants. Thus, we show for the first time that digestive vacuoles of free-living protozoa appear to be an important ecological micro-niche, where gene transfer by conjugation (or retromobilisation) will be favoured. So far, digestive vacuoles have been ignored in genetic and ecological studies. This micro-biotope provides a selective pressure which might enhance the acquisition of virulence genes in cases of mutual interactions between genetically modified micro-organisms and wild-type pathogens. This finding is important for biosafety considerations.

Keywords: Risk assessment; Genetic engineering; Gene transfer; Phagosome; *Tetrahymena*; Environmental microbiology

1. Introduction

Horizontal gene transfer should be considered in risk assessment, when genetically modified micro-organisms (GMM) are released into the environment, either intentionally or accidentally. *Escherichia coli*, one of the most widely used bacteria for the synthesis of recombinant products, may survive longer than expected in river water [1], or sea water [2,3]. It has recently been shown to establish itself in biofilms [4,5]. Even if a significant proportion of the released microbes die or turn into a viable but non-culturable state [2], the greater number of cells will interact with other inhabitants of the ecosystem through the food webs. The majority of released microbes will be ingested by free-living protozoa [6]. Ingestion is most intense on biological surfaces, in sediments or biofilms where the prey settle and nutrients are concentrated [7]. Reported bacteriovory rates for plankton protists range from 200 to 5000 bacteria ciliate^{-1} h^{-1} and from 9 to 266 bacteria flagellate^{-1} h^{-1} [8]. Protozoa can ingest over half the benthic bacteria produced annually, and heterotrophic flagellates can consume 25–100% of the daily bacterial production [8].

* Corresponding author. Tel.: +41 (61) 696 58 01; Fax: +41 (61) 696 69 92; E-mail: bernard.jenni@dm.krinfo.ch

PII S0168-6496(97)00028-7

The free-living ciliate *Tetrahymena pyriformis* is a confirmed laboratory model. It ingests prey by phagocytosis, but can also be readily grown in axenic culture media [9]. Digestive vacuoles (phagosomes) are formed in the oral region (cytopharynx). Phagosomes acquire digestive enzymes from lysosomes. After a digestion period, faecal pellets are egested at the cytoproct and the vacuolar membrane is recycled. The digestive vacuoles are egested in the same order as they are formed and when food is in excess, the rate of phagosome formation equals the rate of egestion [10].

It is important to realise that not all the bacteria ingested will necessarily be killed by the digestive enzymes [11]. Bacteria such as *Salmonella typhi* or *Mycobacterium tuberculosis*, for example, are not digested [12]. Furthermore, several pathogens are able to grow within digestive vacuoles, or escape from them [13]. *Legionella pneumophila* [14,15] and *Listeria monocytogenes* [16] are able to multiply inside protozoa. These invasive human pathogens, which are also able to survive macrophage digestion, may have acquired this ability in free-living protozoa in the course of evolution [17].

We would like to explain now the rationale underlying this work. Thinking in terms of biosafety and risk assessment, horizontal gene transfer is worth studying primarily if scenarios can be envisaged where it equips the recipient with a selective advantage or a new pathogenicity. The digestive vacuoles of protozoa (which would graze on GMM and naturally pathogenic bacteria simultaneously) are natural locations where such scenarios could happen. Thus, observing the behaviour of genetically modified *E. coli* when ingested by protozoa along with ubiquitous wild-type pathogens is unquestionably pertinent to biosafety. One possible scenario might involve the acquisition of pathogenicity genes by GMM, which may then lead to the appearance of unwelcome synergies with some of their specific transgenic properties. Innocuous but genetically modified *E. coli* K12 cells engulfed at the same time as pathogens containing transferable invasive genes might pick up these genes. There is a positive selective pressure towards catching such genes, since they would give the *E. coli* bacterium an obvious advantage in its relation with the grazing ciliate. This hypothesis is supported by the fact that a single invasive gene of *Yersinia pseudotuberculosis* transferred to *E. coli* K12 is sufficient to convert this bacterium into an organism able to invade cultured animal cells [18]. Furthermore, Sansonetti et al. [19] have shown that various levels of pathogenicity were acquired by *E. coli* K12 after stepwise conjugational transfer of a large plasmid and chromosomal segments from *Shigella flexneri*. The recent finding that the structural genes for the cholera toxin are encoded by a replicon related to bacteriophage M13 [20] is also evidence that the emergence of toxigenic *Vibrio cholerae* strains involves horizontal gene transfer. These considerations led us to investigate whether digestive vacuoles or faecal pellets of protozoa might not be an ideal site for gene transfer by conjugation between ingested bacteria.

We studied the transfer of the conjugative plasmid RP4 between laboratory strains of *E. coli* resuspended in tap water and given simultaneously as prey to *T. pyriformis*. *E. coli* K12 and *E. coli* B strains were chosen for the experiments, because K12 derivatives are considered safe hosts for biotechnological applications and B strains are increasingly used in research and development. We determined the duration and efficiency of the digestive cycle and the number of bacterial transconjugants formed within the digestive vacuoles which survived digestion.

2. Materials and methods

2.1. Organisms and culture conditions

Spontaneous nalidixic acid mutants of *E. coli* K12, strain W3110 (DSM 5911), and *E. coli* B (DSM 2840) were grown on tryptose soy agar (TSA) with nalidixic acid (50 μg ml^{-1}). *E. coli* K12 strain J53 (DSM 3876), which contains the broad host-range plasmid RP4, coding for ampicillin, kanamycin and tetracycline resistance, was grown on TSA with kanamycin (50 μg ml^{-1}) at 30°C. The 60 kb conjugative plasmid RP4, belonging to the incompatibility group IncP1, was originally isolated from *Pseudomonas aeruginosa* and is stably maintained in many species at a low copy number [21]. *E. coli* strain BL21(DE3) containing pRSGFP4 (pTU58 modified as pRSGFP4 [22]) expresses a red shift mutant of the

green fluorescent protein (GFP) originating from the jellyfish *Aequoria victoria* [23] under the control of a T7 promoter. This strain was grown on TSA containing ampicillin (50 μg ml^{-1}) and IPTG (1 mM). *Tetrahymena pyriformis* strain GL [24] was cultivated axenically in 1% proteose peptone enriched with 0.1% yeast extract at 25°C [9] and was subcultured every week in T-25 cell-culture flasks.

2.2. Preparation of the protozoa

When the culture reached a density of approximately 10^5 cells ml^{-1}, the protozoa were collected by centrifugation (300×*g*, 3 min, 25°C) and resuspended in membrane-filter-sterilised (0.22 μm) tap water. The protozoa were then incubated at 25°C overnight, which led to an increase in cell size [9] and made them hungry, but not starved. An aliquot of the suspension was diluted with 30 mM NaCl to determine the cell size and number with a CASY cell analyser, Model TTC (Schaerfe System, Reutlingen, Germany).

2.3. Number of digestive vacuoles and duration of digestion

T. pyriformis GL (10^5 cells ml^{-1} in filtered tap water) was fed with *E. coli* BL21(DE3) containing pRSGFP4 at 10^8 bacteria ml^{-1} for 40 min (pulse feeding). These bacteria were monitored by fluorescence microscopy (Leica DMRB, excitation filter 450–490 nm). After that time, the non-ingested bacteria were removed by five centrifugation/washing steps (300×*g*, 3 min, 25°C), using filtered tap water. Then, non-fluorescent *E. coli* cells (DSM 2840) were added to the *T. pyriformis* suspension (chase with alternative prey). The average number of digestive vacuoles per *T. pyriformis* containing fluorescent bacteria was determined by analysing aliquots periodically. The cells were fixed immediately with 2% (v/v) formaldehyde and the number of vacuoles was counted in 170 intact cells by fluorescence microscopy. The number of *T. pyriformis* which did not eat was determined immediately after the centrifugation and taken into account for the calculations.

2.4. Proportion of faecal pellets containing culturable bacteria

To determine the proportion of egested vacuoles (faecal pellets) containing bacteria which survived the digestion phase, *T. pyriformis* was fed for 40 min at 25°C with a 1:1 mixture of *E. coli* K12 strain J53 (DSM 3876) and *E. coli* B (DSM 2840). The mixture was chosen as being similar to that employed in the gene transfer experiment described below. After 40 min of feeding and five centrifugation/washing steps with tap water (300×*g*, 3 min, 25°C), fluorescent latex beads (1 μm, Molecular Probes, No. L-5280) were added to the suspension of protozoa (final concentration of 10^5 cells ml^{-1} in tap water) as alternative prey at a number of 10^3 beads per protozoan. Aliquots were taken after 210 min (corresponding to a complete digestion cycle) and plated on agar slides for subsequent microscopical analysis according to the method described by Schlimme et al. [25]. The proportion of egested vacuoles containing culturable bacteria was determined by examining 340 faecal pellets by phase contrast combined with fluorescence microscopy (Leica DMRB, excitation filter 340–380 nm). Faecal pellets were incubated at 30°C for 30–60 min on nutrient agar slides. Vacuoles located inside lysed bodies of *T. pyriformis*, as well as faecal pellets containing fluorescent beads were not counted.

2.5. Gene transfer by conjugation within digestive vacuoles

The transfer of plasmid RP4 from donor cells (*E. coli* K12, DSM 3876) to *E. coli* K12 (DSM 5911) and *E. coli* B (DSM 2840) recipients was monitored by the appearance of kanamycin resistance. Counter-selection of the donor was made with nalidixic acid. Cells from an overnight agar-plate culture were used to make suspensions of donor and recipients in filter-sterilised tap water. Donor and recipient cell suspensions were added at the same final concentration of approximately 5×10^8 cells ml^{-1} to the prepared population of protozoa (75 ml, 10^5 cells ml^{-1} in a T-75 cell culture flask). After the 40 min feeding pulse, the concentration of the non-ingested bacteria was decreased to about 10^4 bacteria ml^{-1} by five centrifugation/washing steps as described above.

The supernatants from the first and fourth low-speed centrifugations were incubated separately, to periodically determine the frequency of conjugation in the bacterial suspension without protozoa (background levels of conjugation). After the last washing step, the *Tetrahymena* pellets were resuspended in 70 ml sterile tap water. Then, heat-inactivated (70°C, 30 min) *E. coli* cells (recipient strain) were added to a final concentration of approximately 10^8 bacteria ml^{-1} as alternative prey.

Dead bacteria were also added to the supernatants, so as to determine the background frequency of conjugation under similar conditions. The suspensions were then incubated at 25°C and aliquots were taken periodically to determine of the number of transconjugants. This was done by plating aliquots on TSA containing kanamycin and nalidixic acid (50 μg ml^{-1} each). *Tetrahymena* cells lyse when they are plated on agar. Culturable bacteria located within vacuoles or within faecal pellets then begin to grow and form microscopically observable micro-colonies on nutrient agar slides [25], or macro-colonies on agar plates. From each experiment, the regrowth of 40 colonies tested on selective agar was considered as indicative that genuine transconjugants had been obtained.

2.6. Control experiment: inhibition of conjugation by nalidixic acid

To prevent mating on plates during the selection, nalidixic acid was used to select against the donor cells, since it is known that this drug inhibits conjugation [26]. To investigate whether a delay in the inhibition of the conjugation might occur on plates containing nalidixic acid, donor and recipient bacteria were mixed and plated copiously on tap water agar containing nalidixic acid (50 μg ml^{-1}). The plates were incubated at 25°C. Cells were scraped after 0, 15, 30 and 45 min and replated on TSA containing nalidixic acid and kanamycin (50 μg ml^{-1} each).

3. Results

3.1. Digestion cycle of T. pyriformis

It was important to determine the residence time of the vacuoles within the protozoa under the conditions used for the gene transfer experiments. As the protozoa are ingesting prey continuously, a pulse feeding with fluorescent bacteria, followed by a chase with non-fluorescent cells was necessary to monitor one digestion cycle. The number of vacuoles inside *T. pyriformis* which contained fluorescent bacteria was determined by microscopy. The evolution of egestion expressed as a percentage of the number of vacuoles counted (n=2059 within 170 cells) from feeding and preparation time (40 min+20 min) onwards, is reported in Fig. 1a. In order to obtain

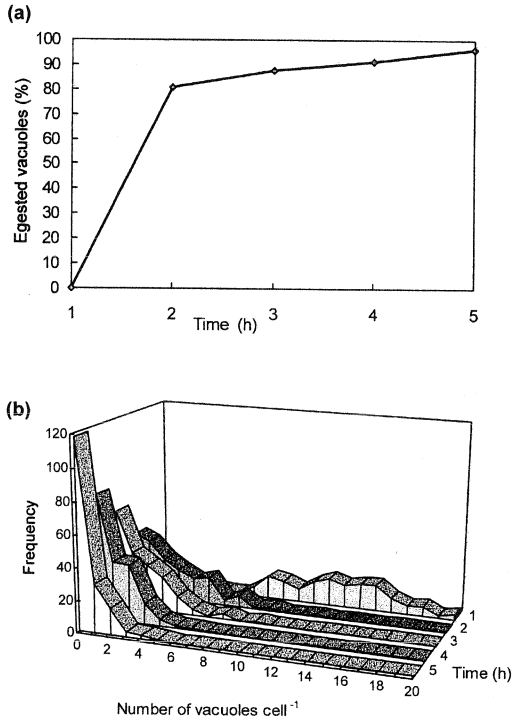

Fig. 1. a: Percentage of vacuoles egested, as a function of digestion time (2059 vacuoles were counted in 170 *T. pyriformis* cells). Eighty percent of the vacuoles were egested within the second hour and more than 90% after 4 h. b: Distribution of the number of vacuoles in *T. pyriformis* cells as a function of the digestion time. An average number of 12–13 vacuoles per cell were found at the beginning of digestion. The curves from a and are based on the same experiment.

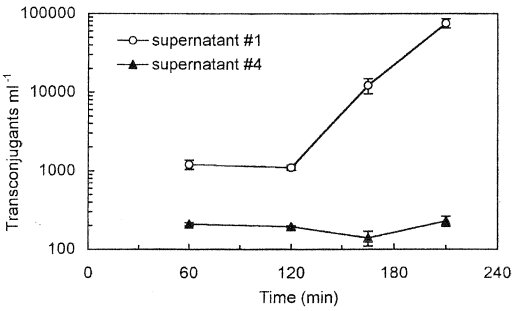

Fig. 2. Determination of the background conjugation. The numbers of transconjugants measured in bacterial suspensions of different cell concentrations (supernatant 1 with 5×10^8 bacteria ml^{-1} and supernatant 4 with 10^4 bacteria ml^{-1}) are reported for the duration of the gene transfer experiment. The number of transconjugants increased only in the concentrated bacterial suspension and only after 2 h of incubation. Supernatant 4 has given the background needed for the gene transfer experiment. Data points represent means of triplicate samples ± standard deviation.

protozoa filled with as many vacuoles as possible, an optimal feeding time of 40 min was set and a further 20 min were required for the centrifugation/washing steps. Consequently, the first determination (1 h) corresponds in fact to vacuoles subjected to the digestion process for 20–60 min. Eighty percent of the vacuoles were egested within the second hour (80–120 min) and more than 90% after 4 h (200–240 min) (Fig. 1a). The distribution frequencies of the number of vacuoles per *T. pyriformis* are presented in Fig. 1b. An average of 12–13 vacuoles per cell was calculated from the first measurement (1 h). Few protozoa did not ingest any prey (6%). Feeding with non-fluorescent cells followed by a chase with fluorescent bacteria gave no significant difference (not shown).

3.2. Viability of the bacteria in faecal pellets

Since we used non-pathogenic bacteria, which have no specific mechanisms to escape digestion, it was important to determine the proportion of faecal pellets which contained viable and still culturable bacteria. Pulse feeding followed by a chase with fluorescent latex beads was a convenient way to monitor the egested vacuoles formed during the first 40 min. The chase was also necessary to avoid the re-ingestion of the faecal pellets by protozoa, since re-ingested bacteria do not survive a second digestive cycle. Ninety-two percent of 340 microscopically analysed faecal pellets formed micro-colonies during incubation on agar slides, thus indicating that they contained one or more viable and culturable bacteria.

3.3. Background conjugation and controls

Since it was not feasible after feeding to separate the non-ingested bacteria completely from the protozoa, it was necessary to keep the conjugation rate in the bacterial suspension as low as possible. For this purpose, five centrifugation/washing steps were necessary to decrease the bacterial concentration from 5×10^8 to 10^4 bacteria ml^{-1}. Moreover, the addition of dead bacteria as alternative prey after the centrifugation/washing steps helped to prevent re-ingestion of faecal pellets by the protozoa, thus avoiding negative artefacts. Dead bacteria were also added to the *Tetrahymena*-free supernatants in order to obtain comparable controls. This procedure also helped to

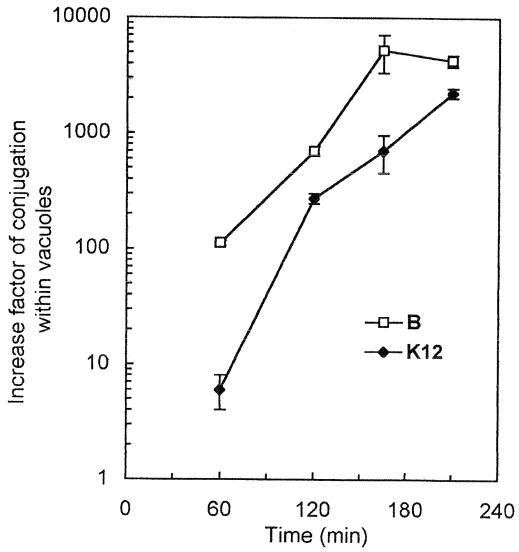

Fig. 3. Increase factor of conjugation within vacuoles and faecal pellets as compared with the background. The data points report the ratio of the total number of transconjugants obtained, in the presence of protozoa, over the number obtained in the supernatant, in the absence of protozoa. After a complete digestion cycle, the increase factor reached 2000 for *E. coli* K12 and 4000–5000 for *E. coli* B. Each curve is based on a different representative experiment, with either *E. coli* K12 or *E. coli* B taken as recipient. Each point represents the mean of three separate samples ± standard deviation. Means of both curves are significantly different at $P<0.05$ with a *t*-test.

maintain a low background of conjugation. The background level depends on the concentrations of recipient and donor cells. The number of transconjugants remained constant (about 200 transconjugants ml^{-1}) in the fourth supernatant (Fig. 2, supernatant 4) which contained 10^4 bacteria ml^{-1}. However, in the supernatant obtained after the first centrifugation (Fig. 2, supernatant 1) which contained 5×10^8 bacteria ml^{-1}, the number of transconjugants began to increase after 2 h. The curves presented in Fig. 2 were drawn from the experiment using *E. coli* K12 as recipient. The values obtained for *E. coli* B taken as recipient are listed in Table 1, with statistical data. In this case, the background was 10-fold lower for the fourth supernatant (about 20 transconjugants ml^{-1}). Nalidixic acid was very efficient in inhibiting gene transfer. We did not obtain any transconjugants in the control experiments carried out in its presence.

3.4. Gene transfer within vacuoles and faecal pellets

The occurrence of gene transfer within vacuoles and faecal pellets has been shown by comparing the evolution of the number of transconjugants obtained in the presence and in the absence of protozoa (Table 1). Fig. 3 shows the ratio of the number of transconjugants found in the presence of *T. pyriformis*, over the background level of transconjugants obtained in the bacterial suspension during a complete digestion cycle. During the first hour of contact between the three microorganisms, the conjugation frequency increases by a factor of 6 for *E. coli* K12 and 100 for *E. coli* B. After a complete digestion cycle, these factors increased to 2000 for *E. coli* K12 and to 4000–5000 for *E. coli* B. The curves shown in Fig. 3 are statistically different ($P < 0.05$), but other experiments would be needed to assert a real biological difference in the behaviour of both strains.

Since the concentration of protozoa (10^5 *Tetrahymena* ml^{-1}) and the total number of internal vacuoles (Fig. 1b) are known, one can calculate the percentage of vacuoles which should contain at least one transconjugant. In our case, the conjugation frequencies per vacuole or per protozoan are more relevant than the frequencies usually reported as transconjugants per recipient or per donor cell. After a digestion period of 210 min with *E. coli* K12 as recipient, the number of transconjugants obtained indicates that conjugation took place in 37% of the

Table 1
Number of transconjugants formed within and outside the vacuoles of *Tetrahymena pyriformis*

	Time (min)	T[a] (CFU/ml)	S.D.[b]	CV[c] (%)
Conjugation outside vacuoles (supernatant 4) with:				
E. coli K12	60	210	10	4.8
	120	195	5	2.6
	165	130	30	23.1
	210	265	35	13.2
E. coli B	60	20	0.5	2.4
	120	20	0.5	2.6
	165	14	3.1	23.0
	210	27	3.5	13.0
Conjugation within vacuoles with:				
E. coli K12	60	1360	331	24.3
	120	47167	5328	11.3
	165	140333	32663	23.3
	210	422000	18833	4.5
E. coli B	60	2077	108	5.2
	120	12667	1247	9.8
	165	106000	5888	5.6
	210	73000	13589	18.6

[a]T: transconjugants (means of three measurements).
[b]S.D.: standard deviation.
[c]CV: coefficient of variation (standard deviation/mean × 100).

digestive vacuoles and/or faecal pellets. With *E. coli* B as recipient, a maximum of 9% of the vacuoles contained a transconjugant. Taking into account that one protozoan usually contains more than 10 vacuoles (Fig. 1b), one can estimate that, statistically, at least one transconjugant was released by each protozoan.

4. Discussion

T. pyriformis requires about 2 h for one digestion cycle and faecal pellets are expelled after that time [27]. This also applies to the conditions we chose for our experiments. Gonzales et al. [11] showed that *T. pyriformis* is able to reduce a bacterial population by a factor 100 per hour. Thus, we were surprised to find that more than 90% of the egested vacuoles still contained viable and culturable bacteria. An indication that non-invasive bacteria are able to survive protozoan digestion was reported by King et al. [14], who showed that coliforms, and not only *Legionella* spp., are able to resist digestion inside protozoa. Several parameters were investigated, which play an important role in the digestion process and in the survival of *L. pneumophila* within digestive vacuoles [15]. A high density of bacteria and the presence of many vacuoles promote the survival of ingested bacteria, but under unfavourable conditions for the bacteria, even pathogens like *L. pneumophila* are not able to multiply within *Tetrahymena*.

Most studies on the fate of ingested bacteria within digestive systems have been performed with terrestrial invertebrates. Feeding the isopod *Porcellio scaber* with a genetically modified strain of *Pseudomonas fluorescens*, Clegg et al. [28] showed that these bacteria were able to survive within the guts and even grow in the faeces. Thus, digestive systems may play an important role in the spreading of released bacteria, the activation of starved bacteria and the promotion of interactions between simultaneously ingested prey. One obvious potential interaction is gene transfer by conjugation.

The occurrence of bacterial conjugation in the gut of nematodes and the recovery of transconjugants in their excrements was confirmed [29]. Transconjugation between strains of *Enterobacter cloacae* was also detected in samples from the digestive tracts and faeces of the cutworm *Peridroma saucia*, but gene transfer was rare [30]. The influence of earthworm activity on gene transfer from *Pseudomonas fluorescens* to soil bacteria was studied and transconjugants from earthworm casts were detected [31]. *E. coli* K12 bacteria were shown to survive a passage through the human intestinal tract and gene transfer occurred without selective pressure (no tetracycline was given to the volunteers) [32]. About 20 transconjugants g^{-1} of faeces were detected 24 h after 10^1 donor bacteria were ingested.

In experiments of the kind mentioned, an underestimation of the number of transconjugants caused by a selection artefact is always possible. We observed that, for example, the choice of antibiotic has a strong influence on the number of detected transconjugants. The use of tetracycline/nalidixic acid instead of kanamycin/nalidixic acid to analyse (as described above) the same cell suspension consistently gave 1000 times fewer transconjugants (not shown). On the other hand, ampicillin/nalidixic acid gave three orders of magnitude more colonies than kanamycin/nalidixic acid. In that case, selection was not effective due to the secretion of β-lactamase into the medium. This annoying artefact was described in detail [33].

For the calculation of the conjugation frequencies per vacuole and per protozoan, we chose the results obtained with the optimal digestion time of 210 min (Fig. 3). Since 90% of the vacuoles were egested by that time, the transconjugants could not stem from poorly digested vacuoles. The incubation time was neither too short nor too long, which would have allowed transconjugants formed during the experiment to multiply and give a false-positive result. Moreover, the fact that 90% of the egested vacuoles contained surviving bacteria indicated that the transconjugants encountered did not stem from a few badly digested vacuoles. We chose *T. pyriformis* GL because it readily forms vacuoles. However, since protozoa filled with many vacuoles tend to eliminate them faster [27], the shorter digestion time may increase the number of viable bacteria egested. It is therefore possible that under natural conditions, the slower digestion might reduce the number of viable transconjugants egested. On the other hand, it was reported that *T. pyriformis* stops feeding below a bacterial density of 10^5 ml^{-1} [34]. It

then starts to swim rapidly around in the search for new places where food is abundant. The chosen experimental procedure, therefore, reflects natural conditions reasonably well.

Returning to biosafety considerations, our results imply that gene transfer to genetically modified *E. coli* (by conjugation) may be effective in digestive vacuoles. A reverse scenario would be the retromobilisation of transgenic constructs by conjugative plasmids into wild-type pathogens. Mobilisation of a recombinant catabolic plasmid by epilithic bacteria was detectable even in oligotrophic conditions [35] and the plasmid RP4 was used to capture non-conjugative plasmids from non-cultivable bacteria [36]. Fifty percent of wild-type strains of *E. coli* were able to promote transfer of pBR-like plasmids in vitro [37]. The widespread utilisation of modern, non-mobilisable plasmid vectors, which lack the basis for mobilisation present in pBR322 (*nic/bom* site) provides a good biological containment [38]. Nevertheless, the recent discovery of conjugative transposons, which would be able to integrate into such plasmids and perform a *cis* mobilisation, suggests that "there is no such thing as a safe plasmid" [39].

To our knowledge, this is the first report on bacterial conjugation within digestive vacuoles of protozoa and their faecal pellets. Since these vacuoles contain transient bacteria that will exhibit specific functions, such sites could be considered genuine ecological micro-niches. A particularity of this micro-biotope (by definition the physical location of a micro-niche) which is relevant for biosafety is its inherent capacity to exert a selective pressure, due to the digestion, towards the acquisition of virulence genes by bacteria.

Acknowledgments

This work was supported by the Swiss National Science Foundation, SPP-Biotechnology, Biosafety Research Module (Grant 5002-035145 to K.H. and B.J.) and by Ciba-Geigy Ltd. W.S. and M.M. were supported by the NSF grant. We thank Robert Peck for the *T. pyriformis* GL strain, Raphael Valdivia for pRSGFP4 and Catherine Fischer for critical reading of the manuscript.

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